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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY 'S DOCKET NUMBER: 2033.000

U S APPLICATION NO. (If known, see 37 CFR 1.5): 09/763720

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/CN99/00126	29 August 1999 (26.08.99)	26 August 1998 (26.08.98)

TITLE OF INVENTION
A PHARMACEUTICAL COMPOSITION CONTAINING CALCINEURIN B SUBUNIT

APPLICANT(S) FOR DO/EO/US Qun WEI, Mingshan YAN, Qinshan GAO, Guohua JIANG, Mulan LIAN, and Yan CHEN

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [x] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [x] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. [ ] The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. [x] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is attached hereto (required only if not communicated by the International Bureau).
   b. [x] has been communicated by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [x] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [x] is attached hereto.
   b. [ ] has been previously submitted under 35 U.S.C. 154(d)(4).
7. [ ] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [x] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). Unexecuted
10. [ ] An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A FIRST preliminary amendment.
14. [ ] A SECOND or SUBSEQUENT preliminary amendment.
15. [ ] A substitute specification.
16. [ ] A change of power of attorney and/or address letter.
17. [ ] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. [ ] Other items or information:

U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/763720	PCT/CN99/00126	2033.000

21. ☐ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**

International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**

International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**

	CALCULATIONS PTO USE ONLY	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 1000.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ --	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	8 - 20 =	–	x **$18.00**	$ --	
Independent claims	2 - 3 =	–	x **$80.00**	$ --	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$270.00**	$ --	
TOTAL OF ABOVE CALCULATIONS =				$ 1000.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.			+	$ --	
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Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$ --	
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a. ☒ A check in the amount of $ 1000.00 to cover the above fees is enclosed.

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NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

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A Pharmaceutical composition Containing Calcineurin B subunit

Technical Field

The present invention relates to a medicament containing Calcineurin subunit B.

Prior Art

Calcineurin (CaN) is the only calcium and calmodulin (CaM)-dependent protein phosphatase as far as we know. It was first discovered and purified from mammal brains in the late 1970s and early 1980s. It was later found that calcineurin also occurs in the non-neural tissues of rabbits and in some cancer cells. CaN, with molecular weight of 80 KD, is a heterodimer consisting of a 61 KD catalytic subunit A and a 19 KD regulatory subunit B (Klee, C. B. et al., The calmodulin regulated protein phosphatase molecular aspects of cellular regulation, 1988, Vol 5, p225).

The subunit A of CaN is the catalytic and regulatory core of the holoenzyme. It includes at least four domains, a metal ions-binding domain (or catalytic domain), a calcineurin B-binding domain, a regulatory effector calmodulin-binding domain and a autoinhibitory domain. The catalytic domain is located on subunit A, which is supported by the fact that subunit A still have the activity of protein phosphatase even if subunits A and B are separated apart. CaN is a serine/threonine protein phosphatase. It was named as protein phosphatase 2B. The amino acid sequence of catalytic subunit A is highly homologuous to other members of the phosphatase family, especially phosphatase 1 and 2A. This homology mainly lies in their catalytic domains, thus their catalytic mechanisms are very similar from each other. Because each phosphatase has special regulatory domain and subunit, thus they are different in structure and function. CaN subunit A differs from other phosphatases in that it have an additional fragment of 170 amino acids containing a subunit B-binding domain, a CaM-binding domain and an autoinhibitory domain in the C-terminus of CaN subunit A, each of them is involved in the regulation of the phosphatase activity of CaN.

The human CaN subunit B is composed of 169 amino acids, and is a member of the calcium-binding protein family. Its primary structure is quite similar to CaM, and troponin C. For example, CaN subunit B and CaM exhibit 31%-35% identity over the amino acid

sequence. Both CaN B and CaM contain four putative EF hand Ca^{2+}-binding sites. Their secondary structures are also quite similar, each protein contains two globular two Ca^{2+}-bingding domains, and two central α-helix which connects the two domains. But there still are some differences between CaN B and CaM. The two calcium-binding domains of CaM are arranged on opposite sides of the central α-helix. However, for CaN B, the two calcium-binding EF-hand domains connect via an α-helix that kinked at Gly-85, and they are arranged linearly along the BBH (subunit B binding helix) of CaN A, together with the amphipathic C-terminal strand, form a hydrophobic groove into which the top half of BBH is embedded. This hydrophobic groove forms the structural basis connecting A and B subunits (Kissinger, C R et al. Nature, 1995, 378: 641; Griffith, J P et al., Cell, 1995, 82: 507). Another difference between CaM and CaN B is that a 14 carbon myristatic acid is connected with the N-terminal glycine residue of CaN B. CaN subunit B does not possess the catalytic activity of protein phosphatase, however, it is highly specific for enzyme activity of the CaN. Although CaN B and CaM function coordinately in the catalytic regulation of CaN A, they play different roles and are irreplaceable between each other. It is known that Ca^{2+} binding to CaN subunit B regulates enzyme activity by lowering Km of the catalytic subunit A for substrate (Perrino, B A et al., J.B.C., 1996, 270: 340).

CaN is a conserved protein, has very broad tissue distribution in eucaryotes. It was found in various tissues including the brain homogenate of human, rat, mouse, pig, frog, fish, and chicken, and non-neural tissues of human and rabbit. CaN B has been found in gastrula crude extract of *Drosophila melanogaster*, but no CaN subunit A was found. A CaM regulated protein phosphatase, found in sea urchin, have the same subunit composition as CaN, and the peptide map of its small subunit is similar to that of CaN B. But the peptide map of its large subunit is different from that of CaN A (Klee, C B et al., Adv. Enzymol., 1987, 61: 149). This indicates that subunit B is more conserved compared with subunit A.

In the studies we have carried out, an anti-mouse CaN antibody was used to detect CaN from human brains and various tumors. The results shows subunit B is present in some tumors such as human brain colloid tumor and meninges tumor in large quantity, but subunit A is hardly detectable. In mouse liver cancer ascites, both A and B subunits can be detected, and there are more subunit B than A (Qun Wei, et al. Chinese Journal of

Biochemistry, 1993, 9: 240). Ongoing studies of the functions of CaN in cancer tissues should be furthered to more deeply understand the disease mechanisms.

Several CaN cDNA clones have been obtained from the brain tissue gene libraries of human, rat, mouse, and rabbit. In eucaryotic organisms, CaN A is encoded by three genes, named as CaN A α, β, and γ, respectively. The so-called A α and A β genes were located on human chromosomes 4 and 10(10q21-q22), respectively. However, CaN B is highly conserved, only one gene was found till now, which is located on chromosome 2(2p16-p15) (Wang, M G et al., Cytogenet. Cell Genet., 1996, 72: 236).

Although the mammal CaN subunit A and B cDNAs were obtained in the late 1980s, how to express them in E.coli in high yield and in active form remains a problem. It has been reported that they can be expressed in insect cells or other cells. Some expressed proteins are in the form of inclusion bodies, thus it was quite difficult to have them extracted and purified. In some reports subunits A and B are coexpressed. We have succeeded in expression of A and B, respectively, in E. coli. To our surprise, the expression level of subunit B is high enough to make this system useful for production of the enzyme in an amount sufficient for isolation, and the purification method was unbelievably simple. Furthermore, the high homology of human, rat, and bovine CaN subunit Bs gives a solid base for pharmaceutical use of this subunit.

CaN has many important biological functions. As the only calcium, calmodulin-dependent protein phosphatase known up to now, it plays an important role in calcium signal transduction and reversible substrate phosphorylation involved in many physiological, pathological processes. Because of its high content in brains, CaN is related to many functions of the brain. More and more attention is being focused on its relation to the ability of learning, memory, and Alzheimer's Disease. Most recently, CaN was found to be the key factor in T-cell activation.

CaN is the key enzyme in T cell activation, which is supported by the fact that it is the common target of different immunosuppressant drugs, such as FK506, CsA, etc. The role of CaN in T cell activation is being elucidated. It was reported that, NF-AT is the substrate of CaN. It was also proved that CaN functions in the way by translocating from cytoplasm to nucleus through binding to NF-AT (Shibasaki, F, et al., Nature, 1996, 382: 370). The mechanism of the function of CaN in nucleus has been elucidated. It binds to the promoter

region of IL-2 gene, which directs the expression of the IL-2 gene. After the IL-2 is excreted out of cells, it binds to the receptor on T cell surface, effecting the proliferation of the T cells.

Most researches which have been carried out directing the biologically function of CaN were focused on its protein phosphatase characteristic, and most experiments were conducted in the presence of both CaN subunits A and B. A few were carried out only in the presence of CaN A or its mutants. There are several reports about the characterization of the biological function of CaN B, especially about the important function of CaN B in the combination of CaN with immunosupressant FK506, CsA and their respective immunophilin FKBP-12, cyclophilin (Li, W. et al., J.B.C., 1993, 268: 14040; Milan D, et al., Cell, 1994, 79: 437; Kawamura A. et al., J.B.C. 1995, 270: 15463; Griffith, JP, et al. Cell, 1995, 82: 507), however, it is believed that this process needs the presence of CaN A. Up to now, there are no reports on the physiological effects by injecting CaN B alone into the body, and there are no reports on CaN B being an anticancer drug and a biological reaction regulator.

The immune state of the body relates closely to the occurrence and development of cancers. Early in 1970, Burnet put forward the theory of immune surveillance, which believes that the immune function evolves as the mechanism of preventing mutation of normal cells to cancer cells, and the mammals recognize and annihilate the cancerized cells primarily through T lymphocytes. In recent years, there are a lot of notable progresses on the research of antigenicity of human and animal cancers, anti-cancer effector cells, and how cancer cells to evade immune surveillance of the body. It is now understood that, except for T cells among the effector cells possessing immune surveillance function, some other cells also participate in this process. The anticancer immune function recognizes not only the cancer related antigens but also the other abnormal phenomenon relating to cancer cells. It is also believed that, cancer is prone to occur as the immune function is injured. Furthermore, recent research indicated that the damage of the immune function is mainly due to the loss of normal immune regulation.

The development of immunology elucidated that, for all the animals including human beings, the immune function is regulated by a rather complicated and quite accurate regulatory system which is called immune regulation complex. Except for the neural and

endocrine system, the entire complex can be divided into two parts: the upward regulation, in which regulatory cells and its secretion products strengthen the immune response; and the downward regulation, in which regulatory cells and its secretion products weaken the immune response. Normally, the immune system reacts exactly to all kinds of stimulation from inside and outside of the body by way of the upper and nether regulations. So the immune system functions at a homeostasis state. Due to some causes like cancer growth, senescence, virus infection, chronic infection, radiological therapy, and the like, the homeostasis is often disturbed or destroyed, so the immune function is greatly weakened. If the immune function is positively regulated (upward regulation), it will be in favor of the control and amelioration of the diseases. The immune regulatory drug exerts the therapeutic function just by regulating the immune function of the body from different aspects. Wherein the immune regulatory substance derived from organism itself is named as Biological Reaction Regulator.

The result of a number of studies showed that, CaN is the key enzyme in T cell activation and also the common in vivo target enzyme of immunosupressant FK506 and CsA. CaN B plays an important role in the binding of immunosuppressant, immunophilin with CaN. CaN B is a highly conserved protein. It has the same amino acid sequence in human, bovine, and rat.

Summary of the Invention

It is one object of the present invention to provide the use of CaN subunit B for the manufacture of medicament for the treatment of mammal diseases by regulating the immune system.

It is another object of the present invention to provide a composition for the treatment of mammal diseases containing effective amount of CaN subunit B or its derivatives possessing CaN B function.

Detailed Description of the Invention

The present invention provides the use of CaN subunit B for the manufacture of medicament for the treatment of mammal diseases by regulating the immune system.

The present invention also provides a composition for the treatment of mammal

diseases, which containing effective amount of CaN subunit B or its derivatives possessing CaN B function.

The composition of the present invention may contain a pharmaceutically acceptable carrier or excipient besides CaN subunit B or its derivatives possessing CaN B function.

The composition of the present invention can be used in the treatment of mammal diseases that can be treated by regulating the mammal immune response, such as cancer, chronic virus infection, senescence, immune depression caused by radiotherapy treatment, and other diseases caused by the disorder of immune function.

The mammals that can be treated with the composition of the present invention can be any mammal, such as human, mouse. The effective amount for treatment of cancer in mouse is 10-200 μ g/day/mouse (for human clinical the effective amount is 100-2000 μ g/kg body weight/day), which can be administered by one dosage or several dosages per day. There are no special restrictions on the route of administration, which may be by vein, or by abdominal cavity. It is preferred to administer successively for many days, for example 5 to 50 days.

The amino acid sequence of above mentioned CaN subunit B is as follows:

1 GNEASYPLEMCSHFDADEIKRLGKRFKKLDLDNSGSLSVEEFMSLPELQQ
51 NPLVQRVIDIFDTDGNGEVDFKEFIEGVSQFSVKGDKEQKLRFAFRIYDM
101 DKDGYISNGELFQVLKMMVGNNLKDTQLQQIVDKTIINADKDGDGRISFE
151 EFCAVVGGLDIHKKMVVDV

For CaN B derivatives obtained by the addition, deletion, substitution of one or more amino acids in the above mentioned sequence, or the functional derivatives obtained by chemical modification of the side chains of one or more of the amino acids, can also be used in the composition of the present invention, so long as it remains the biological activity of CaN subunit B.

In vitro experiments demonstrated that, CaN B functions by directly acting on spleen lymphocytes in rats, making them to proliferate 4-7 times. CaN B and mitogen Con.A obviously act synergistically in the stimulation to lymphocytes. 62.5 μ g of CaN B can reverse 88% of the immune inhibitory effect of cortisone. In vivo experiments in mouse

also indicated that CaN subunit B can accelerate the augmentation of spleen which is an important immune organ. This result proved that CaN subunit B is an excellent immune upward regulation agent or an excellent biological response regulator.

The in vivo anticancer effect of CaN subunit B is significant, it can remarkably reduce the ascites of H22 liver cancer mouse, and can prolong the life by 50-83% of the mouse suffering from cancer. With the dosage as above, CaN B can inhibit the tumor growth of S180 solid tumor by more than 57%. The difference is statistically significant compared with the control group.

The acute toxicity experiment showed that the toxicity of CaN B is negligible. No mouse died in 24 hours during the acute toxicity experiment when 4-50 times of the effective dosage was administered.

The present invention is based on the fact that we have expressed CaN subunit B in E. coli in high level and purified it efficiently from lysate. We have used CaN B directly or its functional derivatives as a drug in the treatment of disease of mammal.

Examples

Example 1. Expression plasmid construction and protein expression

CaN subunit B cDNA was obtained from rat brain cDNA library (Perrino B et al., J. Biol. Chem., 1996, 270: 340). Forward primer was designed as 5'-CCGCCATATGGGAAATGAGGCGAGTT-3', reverse primer was designed as 5'-CGCGGGATCCTCACACATCTACCACCA-3'. After PCR amplification, the expected CaN B gene cDNA fragment purified from agarose gel and pET21a vector were double-digested with restriction enzymes Nde I and BamHI, ligated with T4 DNA ligase and transformed into BL21(DE3) plysS *E.coli*. The positive clones were kept at 4℃ in LB solid medium containing 50ug/ml Amp. 1 liter of TM medium containing 50 μ g/ml Amp was inoculated with 5-10 ml freshly grown culture. The culture was incubated 5-6 hrs in an air shaker at 37℃, 250 rpm. The cells from the above culture were spun down at 5000 x g for 20 minutes at 4℃. After discarding the supernatant, the cell pellet were stored at -20℃.

Example 2. Construction and expression of CaN subunit B splicesome and mutants

The N- and C-terminal CaN B splicesomes were obtained through PCR amplification using specifically designed primers. Non-N terminal and non-C terminal mutants construction was performed by pAlter mutation plasmid system (Promega). The expression system was the same as wild-type CaN subunit B.

Example 3: The preparation of CaN subunit B

1. Breaking Cells: The cell pellet from 1 liter culture was resuspended in 50-100 ml ice-cold cell lysis buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.2 mM PMSF, 1 mM β-mercaptoethanol), The cells were broken by sonication (200 W, 2-3 minutes).

2. Protein extraction and purification: After the cells were broken, the mixture was kept at 100℃ boiling water for 30-40 minutes, the supernatant was obtained by centrifugation at 12,000 x g for 20 minutes. The supernatant was CaN subunit B crude extract. The hydrophobic Phenol-Sepharose CL-4B column was used for purification of CaN subunit B. After the column was equilibrated with equilibration buffer(20 mM Tris-HCl, pH 7.4, 0.5 mM $CaCl_2$, 1 mM β-mercaptoethanol), the extract (added 3 mM $CaCl_2$, 1 mM β-mercaptoethanol) was slowly loaded onto the column. No less than 10 bed volumes of the equilibration buffer was required to thoroughly wash the column. After washing, the target protein was eluted from the column using the elution buffer(20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5 mM DTT). About 120 mg electrophoretically pure protein was obtained from 1 liter culture. The purified protein was lyophilized and stored at -20℃.

Example 4: Detection, identification, quality control and method of use

1. DNA sequencing: The cDNA contains 507 nucleotides, the sequence of which is the same as the reported CaN subunit B cDNA sequence.

2. Amino acid sequencing: The 10 N-terminal amino acids of CaN B are GNEASYPLRM. This indicated that expressed protein is the human CaN subunit B protein as reported. Both rat and human have the same amino acid sequence of CaN B.

3. Purity analysis: Over 98%, which is shown by SDS-PAGE.

4. Identification of physical and chemical characteristics: IP=4.8, standard absorbance $\varepsilon_{277nm}^{1\%}$=3.1.

5. Concentration analysis: UV-spectrophotometric method.

6. Identification experiment: Positive result in Western Blot using anti-CaN subunit B antibody; UV-spectrum.

7. Biological activity: Activation of CaN subunit A.

8. Characters and Method of use: CaN subunit B is highly soluble in water, the water solution is colorless and quite clear. Soluble in physiological saline or pH-neutral buffer(mannitol can be added), CaN subunit B can be kept at –20 ℃ for over 2 years.

Example 5, In vivo anti-cancer experiments of CaN B

1. Prevention experiment, life prolongation experiment, and dose experiment of H22 liver ascites cancer:

Materials and method: BAL B/C mouse, ♂,weight 20±1 g. The mouse of experiment group were injected (i.p.) CaN subunit B 10, 100, or 200 ug/0.2ml/day/mouse, the mice of control group were injected (i.p.) PBS 0.2 ml/day/mouse. After two days the mice were inoculated 1 X 10^6 H22 ascites cancer cells, and at the same time, CaN B or PBS was injected(totally for 7 days). The living time of each mouse was recorded after inoculation. The life prolongation rate was calculated according to the formula as follows, and the result is shown in Table 1 and 2 below.

$$\text{Life prolongation rate} = \frac{\text{Average living days of experiment group - Average living days of control group}}{\text{Average living days of control group}} \times 100\%$$

Table 1

Groups	Average living days±SD	Life prolongation rate	t-test
Control	19.5±0.76		
10 μg CaN B	24.6±3.35	26%	<0.2
100 μg CaN B	29.2±1.36	50%	<0.001

Table 2

Groups	Average living days±SD	Life prolongation rate	t-test
Control	18.75±0.5		
200 μg CaN B	34.25±4.6	82.7%	<0.001

2. Treatment experiment of H22 liver ascites cancer, life prolongation experiment:

Materials and method: BAL B/C mouse, ♂, weight 20±1 g, were inoculated (i.p.) with 1 X 10^7 H22 ascites cancer cells. The mice were randomly divided into two groups: experiment group, which was injected with CaN subunit B of 200 μg/0.2ml/day/mouse; control group, which was injected with PBS of 0.2 ml/day/mouse. The living days after inoculation were recorded, and the result is shown in Table 3 below.

Table 3

Groups	Average living days±SD	Life prolongation rate	t-test
Control	19.0±1.6		
Experiment	29.0±8.9	52.6%	<0.025

3. Treatment experiment of S180 solid cancer:

Materials and method: Kunming White mouse, ♂, weight 20±1 g, was inoculated (i.e., at armpit) with 1 X 10^7 S180 ascites cancer cells (about 0.2 ml/mouse). The inoculated mice were randomly divided into two groups. After four days (when the tumor was visible), the control group was injected with PBS of 0.2 ml/day/mouse, and the experiment group was injected with CaN subunit B of 100 ug/0.2ml/day/mouse (totally for 7 days). After 24 hours from the latest injection, the mice were killed and the solid tumor and spleen were removed from the body, and were weighted. The tumor inhibition rate was calculated according to the formula as follows and the result is shown in Table 4 below.

$$\text{Tumor inhibition rate} = \frac{\text{Average tumor weight of control group- Average tumor weight of control group}}{\text{X100\%}}$$

Average tumor weight of control group

Table 4

Groups	Average spleen weight±SD	Weight increase rate	t-test	Average tumor weight±SD	Inhibition rate	t-test
Control	0.25±0.09			1.38±0.66		
Experiment	0.32±0.08	25.5%	<0.1	0.59±0.39	57.1%	<0.01

Example 6: In vitro cell biological immune experiments of CaN B

1. The direct effect of CaN subunit B on rat spleen cell proliferation:

Materials and method: Rat spleen lymphocytes were obtained by using conventional method, then were suspended in 1640 culture medium at a concentration of 5 X 10^6 cells/ml. Each well of 96 well plate contained 5 X 10^5 cells in 0.2 ml, and CaN subunit B was added at the concentration of 0, 2.5, 12.5, or 62.5 μg/ml. The cells were cultured for 48 hrs at 37 ℃, 5% CO_2. Then ^{3}HdR (0.5 μ Ci/well) was added, and the cells were cultured for 8-16 hrs. The cultured cells were collected onto a glass fiber membrane using multiple-head cell collector. After the membrane was dried, scintillation solution was added, and cpm value was obtained by using a scintillation counter. Then the difference between before and after addition of CaN subunit B was obtained. The difference was showed in the form of proliferation times calculated according to the formula as follows, and the result is shown in Table 5 below.

$$\text{Proliferation Times} = \frac{\text{Average cpm value of CaN B group}}{\text{Average cpm value of control group}} \times 100\%$$

Table 5

Dosage	cpm±SD	Proliferation times	t-test
0(control)	467±81		
2.5 μg CaN subunit B	375±25		
12.5 μg CaN subunit B	1704±194	3.7	<0.001
62.5 μg CaN subunit B	3041±390	6.5	<0.001

2. The synergistic effect of CaN subunit B and Con.A in the proliferation of rat spleen lymphocytes:

The materials and method are the same as that in part 1 of example 6. At the same time of adding CaN subunit B, the immune stimulator Con.A was also added at a concentration of 5 μg/ml. The synergistic effect of CaN subunit B and Con.A in the proliferation of rat spleen lymphocytes was clearly seen from the result as shown in Table 6 below.

Table 6

Dosage	cpm±SD	Proliferation times	t-test
0(control)	158000±3200		
2.5 μg CaN subunit B	155000±7000		
12.5 μg CaN subunit B	174000±5200	10.2%	<0.001
62.5 μg CaN subunit B	192000±3600	21.8%	<0.001

3. The reverse effect of CaN subunit B in the immune inhibition of cortisone:

The materials and method are the same as that in part 1 of example 6. On a 96-well microtiter plate, after the cell culture was added, immune stimulator Con.A was added at a concentration of 5 μg/ml, then cortisone (10^{-7} mM/L) and thereafter CaN B was added. The result of reverse effect of CaN subunit B on the immune inhibition of cortisone was shown in Table 7 below.

Table 7

Dosage	cpm±SD	Proliferation reverse rate	t-test
0(control)	66400±4500		
2.5 μg CaN subunit B	63500±2800		
12.5 μg CaN subunit B	73900±4000	19.4%	<0.001
62.5 μg CaN subunit B	125000±5100	87.9%	<0.001

Example 7: Acute toxicity experiment of CaN subunit B on mouse

Animals: BAL B/C mouse, ♂, weight 20±1 g.

Results:

Table 8

Groups (dosage)	Number of animals	Number of died animals		
		0-24 hrs	24-48 hrs	48 hrs-1 month
4 times of normal dosage (0.4mg/0.2ml/mouse)	4	0	0	0
20 times of normal dosage (2mg/0.2ml/mouse)	4	0	0	0
50 times of normal dosage (5mg/0.2ml/mouse)	8	0	3	0

Claims

1. A composition for treatment of mammal diseases, comprising an effective amount of CaN subunit B or its derivatives possessing CaN B function.

2. The composition of claim 1, wherein the treatment is achieved by regulating the immune system of the mammal.

3. The composition of claim 1, wherein the composition contains a pharmaceutically acceptable carrier or excipient.

4. The composition of claim 1, wherein the mammal disease to be treated is a disease which can be treated by regulating the immune response of the mammal.

5. The composition of claim 1, wherein the mammal disease to be treated is a cancer disease of mammal.

6. The composition of claim 1, wherein said mammal is human.

7. The composition of claim 1, wherein, said mammal is mouse, the effective amount for the treatment of tumor disease is 10-200 μg/day/mouse.

8. Use of CaN subunit B for the manufacture of medicament for the treatment of mammal diseases.

Abstract

The present invention provides a pharmaceutical composition for treatment of mammal disease by regulating the immune system, which contains CaN B subunit and/or its functional derivant in effective amount for treating the disease.

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63) and POWER OF ATTORNEY

☐ Declaration Submitted with Initial Filing
OR
☑ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16(e)) required)

Attorney Docket Number: 2033.000
First Named Inventor: Qun WEI et al.
COMPLETE IF KNOWN
Application Number: 09/763,720
Filing Date: _____
Group Art Unit: _____
Examiner Name: _____

As a below named inventor, I hereby declare that:

My residence, mailing address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Pharmaceutical Composition Containing Calcineurin B Subunit

the specification of which
☑ is attached hereto
and
☑ was filed on August 29, 1999 as PCT International Application Number PCT/CN99/00126.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to the patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)–(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed	Certified Copy Attached?
98 117642.9	China	August 26, 1998	☐	☐ Yes ☐ No
(Number)	(Country)	(Foreign Filing Date)		
			☐	☐ Yes ☐ No
(Number)	(Country)	(Foreign Filing Date)		
			☐	☐ Yes ☐ No
(Number)	(Country)	(Foreign Filing Date)		

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

(Application Number)	(Filing Date)	☐ Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.
(Application Number)	(Filing Date)	

DECLARATION – Utility or Design Patent Application and POWER OF ATTORNEY

As a below-named inventor, I hereby appoint the registered practitioners named below as my/our attorney(s) or agent(s) to prosecute this application, and to transact all business in the Patent and Trademark Office connected therewith:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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DECLARATION – Utility or Design Patent Application and POWER OF ATTORNEY

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Inventor's Signature: _____ Date: _____

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Mailing Address: _____

(city, state, zip, country): _____

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